Doxycycline therapy for abdominal aneurysm: Improved proteolytic balance through reduced neutrophil content

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Background: **Matrix metalloproteinase-9 (MMP-9) is thought to play a central role in abdominal aortic aneurysm (AAA) initiation. Doxycycline, a tetracycline analogue, has direct MMP-9-inhibiting properties in vitro, and it effectively suppresses AAA development in rodents. Observed inhibition of AAA progression, and contradictory findings in human studies evaluating the effect of doxycycline therapy on aortic wall MMP-9, suggest that the effects of doxycycline extend beyond MMP-9 inhibition and that the effect may be dose-dependent.**
Methods: **This clinical trial evaluated the effect of 2 weeks of low- (50 mg/d), medium- (100 mg/d), or high-dose (300 mg/d) doxycycline vs no medication in four groups of 15 patients undergoing elective AAA repair. The effect of doxycycline treatment on MMP and cysteine proteases, and their respective inhibitors, was evaluated by quantitative polymerase chain reaction, Western blot analysis, immunocapture protease activity assays, and immunohistochemistry.**
Results: **Doxycycline was well tolerated and no participants dropped out. Doxycycline treatment reduced aortic wall MMP-3 and MMP-25 messenger RNA expression ($P < .045$ and $P < .014$, respectively), selectively suppressed neutrophil collagenase and gelatinase (MMP-8 and MMP-9) protein levels ($P < .013$ and $<.004$, respectively), and increased protein levels of the protease inhibitors tissue inhibitor of metalloproteinase 1 and cystatin C ($P < .029$). As for the apparent selective effect on neutrophil-associated proteases, we sought for a reducing effect on aortic wall neutrophil content that was indeed confirmed by immunohistochemical analysis that revealed a 75% reduction in aneurysm wall neutrophil content ($P < .001$).**
Conclusions: **Independent of its dose, short-term preoperative doxycycline therapy improves the proteolytic balance in AAA, presumably through an effect on aortic wall neutrophil content. This study provides a rationale for doxycycline treatment in patients with an AAA as well as in other (vascular) conditions involving neutrophil influx such as Kawasaki disease and Behçet disease. (J Vasc Surg 2009;49:741-9.)**

Clinical Relevance: **The concept of pharmaceutical stabilization of abdominal aneurysms is promising. Doxycycline, a tetracycline analogue, is considered a lead candidate, but its mode of action is still unclear. This clinical trial showed that doxycycline treatment, through a profound effect on the number aortic wall neutrophils, has a pronounced but selective effect on the proteolytic balance in the abdominal aneurysm, as indicated by reduced matrix metalloproteinase (MMP)-8 and -9 levels and concentrations of tissue inhibitor of metalloproteinase-1 and cystatin C. The observation that doxycycline has a selective effect on neutrophil-derived proteases is remarkable and novel, and suggests that doxycycline may also be effective in other vascular conditions involving neutrophils, such as Kawasaki disease and Behçet disease, and nonvascular conditions such as chronic obstructive pulmonary disease.**

An aneurysm of the abdominal aorta (AAA) is a common pathology and a major cause of death due to rupture.[1] Risk of rupture is negligible in small AAAs, but increases exponentially in AAAs with a diameter of ≥55 mm.[1] Hence, current approaches toward AAAs are surveillance of small AAAs and preventive surgical repair of larger AAAs (ie, ≥55 mm).[2] Unfortunately, conventional transabdominal (open) repair is associated with considerable morbidity and mortality.[3] Although short-term results of endovascular repair appear more favorable, rates of mid- and long-term mortality are similar to those of conventional repair.[4,5] Endovascular repair, moreover, requires life-long follow-up, and the number of reinterventions after endovascular repair is high (approximately 9%), although most are minor.[6] As a consequence, the effectiveness of endovascular AAA repair is now being challenged.[7,8]

Pharmaceutical therapy inhibiting aneurysmal growth, and thus reducing the need for invasive treatment, could have major advantages for patients as well as socioeconomic benefits.[9] Excess matrix degradation that is not balanced by matrix deposition is considered pivotal to aneurysmal growth.[1] This led to the proposal that pharmaceutical

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intervention that would reduce protease activity, in particular, matrix metalloproteinase-9 (MMP-9), could restore the balance between matrix degradation and deposition and thus reduce aneurysmal growth.[10,11]

Members of the tetracycline family of antibiotics have been recognized as inhibitors of MMP expression and activity. It was thus proposed that doxycycline, a tetracycline analogue, may reduce excess MMP-9 activity in AAA and constitute a pharmaceutical means of reducing aneurysmal growth.[10,11] Indeed, doxycycline has been shown to inhibit aneurysm formation in various animal models of AAA.[10-13] Moreover, results from two preliminary phase I/II studies in patients under surveillance for AAA suggest that long-term doxycycline treatment may also attenuate or even forestall aneurysm growth.[14,15]

Despite these promising findings, a number of questions, in particular the mode of action of the drug,[16] remain to be answered. The rationale behind doxycycline therapy was to prevent aneurysmal growth through inhibition of elastolysis mediated by MMP-9.[10,17] Yet, it is now recognized that loss of elastin is a very early event in AAA formation and that aneurysmal growth and ultimate rupture essentially depend on loss of structural collagens.[1,18] However, MMP-9 is a gelatinase that can only degrade collagen after initial cleavage by specific collagenases.[18] Observed inhibition of aneurysmal growth in animal models and human studies therefore suggests that apart from its effects on MMP-9, doxycycline therapy also influences the collagenases.

A recent study[19] evaluated the effect of preoperative doxycycline treatment on messenger RNA (mRNA) expression of the collagenases MMP-1, MMP-13, and MMP-14, but failed to observe an effect on these proteases. The effects on the primary collagenases in AAA (MMP-8 and the cysteine proteases cathepsins K, L, or S)[18] were not evaluated.

Another issue that has not been addressed is the contradictory results of human studies evaluating the effects of doxycycline on MMP-9 expression and activity. Although the initial human study showed that preoperative doxycycline reduced MMP-9 mRNA expression and activity,[17] a later more elaborate study failed to observe such an effect.[19] The basis for these divergent findings is unclear, but they may reflect increased MMP-9 expression as result of an immunostimulatory effect on monocytes as seen at higher doses of tetracyclines[20]; indicating that the contrasting findings in human studies may reflect a dose–response relationship.

To evaluate the effects of doxycycline therapy on the MMP and cysteine proteases,[18] their respective inhibitors,[18,21] and to test whether the apparent contradictory findings of human studies on MMP-9 relate to dose-dependency, we evaluated the effect of low (50 mg/d) regular (100 mg/d) and high (300 mg/d) doses of doxycycline on the members of the MMP family of proteases and the cysteine collagenases cathepsin K, L, and S. Results from this study show that, irrespective of dose, 2 weeks of preoperative doxycycline treatment improves the proteolytic balance through reduction of the neutrophil-associated proteases MMP-8, MMP-9 and MMP-25, and an increase of the protease inhibitors tissue inhibitor of metalloproteinase 1 (TIMP1) and cystatin C. Further evaluation of this phenomenon showed that doxycycline therapy profoundly reduced the number of infiltrating neutrophils in the aneurysmal wall.

METHODS

Patients. The study was performed on intention-to-treat basis. The study included 60 patients scheduled for elective, open aneurysm repair and excluded patients with chronic inflammatory disease or inflammatory aortic aneurysms. Decisions for open repair were based on anatomic (eg, neck, elongation) and patient characteristics (age), and on patient preferences. Patients were randomly assigned to receive 50, 100 or 300 mg doxycycline per day, or no medication (control group), with 15 patients in each group. In two patients in the low-dose doxycycline group, the operation had to be postponed because of full occupancy of the intensive care unit; therefore, 13 patients were evaluated in this group. Medication was started 14 days before the planned operation, and the last dose was taken in the evening before surgery. Postoperative outcome was similar in all groups.

Procedure. The aneurysm sac was opened and adhering thrombus was manually removed. A tissue sample was obtained from the anterior-lateral aneurysm wall at the maximum diameter of the aneurysm. Wall samples were immediately halved. One half was snap-frozen in carbon dioxide-cooled isopentane or liquid nitrogen and stored at −80°C for later analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensens solution, 120 hours), and paraffin-embedded for histologic analysis. All analyses were performed in a researcher-blind fashion.

For comparison of AAA wall with nonaneurysmal wall, we used nonaneurysmal aortic wall samples from brain-dead kidney donors. Only patches displaying advanced atherosclerotic lesions (equalling the characteristics of grade IV-VI lesions by the Stary classification)[22] were selected. The nonaneurysmal control group comprised 11 patients (7 men, 4 women) aged 55.6 ± 10.2 years, with an aortic diameter of <2.0-cm. All nonaneurysmal control samples were obtained from the level of the renal artery and during a laparotomy (ie, from a comparable region and during a similar procedure as the AAA samples).

The study was approved by the Medical Ethical Committee of the Leiden University Medical Centre (LUMC), Leiden, The Netherlands.

RNA isolation and real-time quantitative polymerase chain reaction. RNA isolation and quantitative mRNA analysis by LightCycler Real-time polymerase chain reaction (PCR, Applied Biosystems, Nieuwerkerk aan den Ijssel, The Netherlands) were performed by following previously published protocols.[21] All mRNA data were standardized on basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

Tissue homogenization. Aortic wall tissues were pulverized in liquid nitrogen and homogenized in lysis buffer (10mM Tris, pH 7.0; 0.1mM calcium chloride, 0.1M sodium chloride, 0.25% (v/v) Triton X-100). This protocol releases both soluble as well as membrane-bound proteases. Samples were subsequently centrifuged at 13,000 rpm for 10 minutes at 4°C, snap-frozen, and stored at −80°C until use. Homogenates were standardized according to their protein content (Pierce, Rockford, Ill).

Specific immunocapture MMP activity assays. MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and MMP-14 activity assays (Amersham Biosciences, Buckinghamshire, United Kingdom [UK]) were performed according to the supplier's recommendations. In these assays, target proteases are captured by a specific antibody immobilized on microtiter plates, and the proteolytic activation of a modified proenzyme by the captured protease is used to quantify the activity of the captured protease. These assays allow sensitive and specific assessment of active MMPs, as well as pro-MMP upon activation of latent MMP by a mercuric salt (p-aminophenylmercuric acetate) in in vitro systems.

Western blot analysis. Western blot analysis was used to quantify the amount of proenzyme as well as the preceding protease activation through quantification of the amount of activated protease that was present in the tissue homogenates as protease-inhibitor complex.[18] Preliminary analyses showed that the antibodies used allow evaluation of both pro and active forms of the respective proteases and that the standard denaturing conditions required for Western blot analysis result in full dissociation of MMP–TIMP and cathepsin–cystatin C complexes, thus showing that these analyses allow assessment of inhibitor-bound MMPs and cathepsins.[18]

Western blot analyses for the proteases, as well as for cystatin C and TIMP1, were performed as previously described[23] using the antibodies antihuman MMP-2 (PC-158, the Bindingsite, Birmingham, UK), anti-MMP-3 (PC-112, the Bindingsite), anti-MMP-7 (RP1MMP7, Triple Point Biologics, Cambridge, UK), anti-MMP8 (MAB3316, Chemicon, Chemicon Europe Ltd, Chandlers Ford, UK), anti-MMP-9 (TNO-BEA-21, TNO, Leiden, The Netherlands), anti-cathepsin K (IM55L, Calbiochem, Breda, The Netherlands), anti-cathepsin L (AF952, R&D Systems, Abingdon, UK), anti-cathepsin S (sc-6505, Santa Cruz, Heerhugowaard, The Netherlands), anti-cystatin C (sc-16989, Santa Cruz), and anti-TIMP1 (AB8229, Chemicon). The amount of protein in all samples was standardized using actin levels (anti-actin sc-1615, Santa Cruz).

All secondary antibodies were obtained from Santa Cruz Biotechnology (Heerhugowaard, The Netherlands). Immunoblots were visualized using Super Signal West Dura Extended Duration Substrate (Perbio Science, Etten-Leur, The Netherlands), and a luminescent image workstation (UVP, Cambridge, UK). LabWorks 4.6 software (UVP, Upland, Calif) was used to quantify the immunoblots.

Immunohistochemistry. Immunohistochemistry was performed using 4-μm deparaffinized, ethanol-dehydrated tissue sections. Sections were incubated overnight with a myeloperoxidase (A 0398 DAKO, Heverlee, Belgium), a MMP-8 (Medix Biochemica, Milsbeek, The Netherlands), or a CD68 (M 0718 DAKO) antibody. Sections were stained with Nova Red (Vector Laboratories, Burlingame, Calif) and counterstained with Mayer hematoxylin. Controls were performed by omitting the primary antibody. Specificity of myeloperoxidase staining for neutrophils was validated by morphometric analysis of myeloperoxidase-positive cells by an experienced vascular pathologist (Dr J. H. von der Thüsen). Slides were examined by two independent observers who were unaware of the patient's status.

Statistical analysis. Expression of mRNA was compared by *t* test or Wilcoxon Mann-Whitney *U* test, where appropriate. Results of the Western blots and immunohistochemistry were analyzed by Wilcoxon- Mann-Whitney *U* test to compare different groups. Statistical significance was accepted at a value of $P < .05$. Many findings in this study reflect correlated data, and as such a Bonferroni correction was *not* applied for the data in this study. Yet, a Bonferroni correction should be considered when interpreting the results of uncorrelated data. Possible dose–response relationships were evaluated by quadratic regression analysis. All analyses were performed using SPSS 12.0.1 software (SPSS Inc, Chicago, Ill).

RESULTS

Patients. Clinical characteristics of the patients are compiled in the Table. All four groups were comparable with regard to age, sex, pharmaceutical treatment, and AAA diameter. Owing to the reticence toward statin use in The Netherlands, <10% of the patients in the study were receiving statin therapy. Doxycycline treatment was well tolerated, and no patients dropped out.

Effects of doxycycline treatment on MMP expression and activation. Compared with nonaneurysmal control aorta wall, AAA tissue was characterized by increased mRNA expression of soluble MMP-3 ($P < .029$) and MMP-9 ($P < .001$), as well as by abundant expression of the membrane type MMPs, MMP-16 ($P < .016$) and MMP-25 ($P < .001$). Expression of all other proteases and their inhibitors was similar in control tissue and AAA (Supplementary Table I, online only).

Fig 1 shows that independent of its dose, 14 days of preoperative doxycycline therapy attenuated MMP-3 ($P < .045$) and MMP-25 ($P < .014$) mRNA expression. All three doses of doxycycline increased MMP-8 mRNA expression to values comparable to those found in control aorta (Supplementary Table II, online only). Expression of all other MMPs, including MMP-9, was not influenced by doxycycline treatment (Supplementary Table II, online only).

Possible effects of doxycycline treatment on the post-transcriptional regulation of MMP protein levels and activity were evaluated by specific protease activity assays and Western blot analyses. Direct measurement of active MMPs in aortic tissue is not feasible because of the rapid inactivation of the active enzymes by the endogenous inhibitors[18];

Table. Patient characteristics

Variables[a]	*Control AAA*	*Doxycycline*		
		50 mg	*100 mg*	*300 mg*
Evaluable patients, No.	15	13	15	15
Age, mean (range) y	74.8 (69-84)	72.7 (62-85)	74.1 (50-88)	72.1 (58-87)
AAA diameter, mean cm	6.7	6.5	6.3	6.7
Time between diagnosis and surgery, mean mon	6	7	5	4
Female sex, No.	1	2	2	3
Current smoker, No.	6	6	7	5
Medication use, No.				
Statin(s)	1	1	1	2
Antihypertensive(s)	8	7	8	7
Antiplatelet therapy	10	8	8	8

AAA, Abdominal aortic aneurysm.
[a]Values of *P* for all variables were not significant.

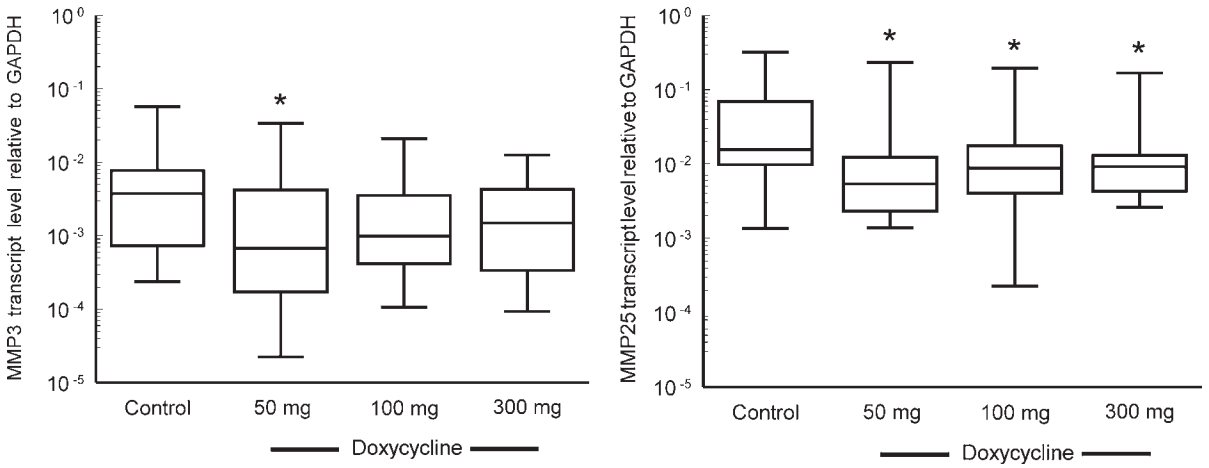

Fig 1. Left, Matrix metalloproteinase *(MMP)*-3 and **(right)** MMP-25 transcript levels relative to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. Independent of its dose, doxycycline reduces aortic wall MMP-3 and MMP-25 messenger RNA expression. Maximum level of suppression was already found in the low-dose doxycycline group. Results for the higher doses are more variable. Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range *(bars)*. *$P < .045$ and $P < .014$ respectively (*t* test).

therefore, MMP activities in the protease activity assays were only measured after in vitro activation of latent proenzymes. Results from these assays showed a maximum level of suppression of MMP-2, MMP-8, and MMP-9 proenzyme levels in the low-dose doxycycline group (MMP-2, $P < .015$; MMP-8, $P < .013$; and MMP-9, $P < .004$; Fig 2). Results for the higher doses were more variable and showed reduced aortic wall MMP-8 proenzyme levels in the standard-dose (100 mg), and reduced MMP-9 proenzyme levels in the high-dose (300 mg) doxycycline group ($P < .020$; Fig 2). MMP-1 and MMP-13 proenzyme levels both remained below the detection limit of the protease activity assays.

We performed Western blot analysis to evaluate a possible effect of doxycycline treatment on protease activation. Fig 3 shows that doxycycline treatment was associated with a dose-independent reduction of MMP-8 and MMP-9 *activation* ($P < .004$). Findings for MMP-8 proenzyme paralleled results of the protease activity assays and showed reduced MMP-8 proenzyme levels for all doses of doxycycline ($P < .010$). However, results for MMP-9 proenzyme were not statistically significant in the low-dose and normal-dose doxycycline groups ($P < .073$; Fig 3). MMP-2, MMP-3, and MMP-7 proenzyme and active enzyme levels were not influenced by doxycycline treatment (Supplementary Table III, online only). This indicates that doxycycline treatment neither influenced protein levels nor activation of these proteases.

Doxycycline treatment: cysteine protease expression and activation. Doxycycline treatment for 14 days did not influence mRNA expression of the cysteine proteases cathepsin K, L, and S (Supplementary Table II, online only). Western blot analysis for pro and activated forms of cathepsin K, L, and S showed that doxycycline therapy did not influence cysteine proenzyme levels or their activation (Supplementary Table III, online only).

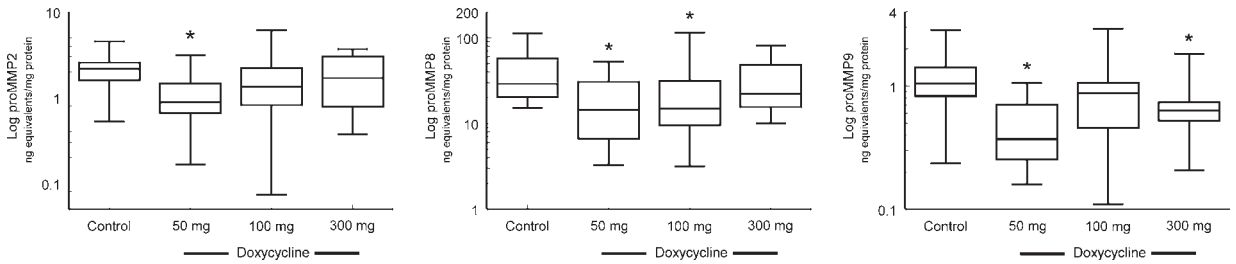

Fig 2. The effect of doxycycline on **(left)** matrix metalloproteinase *(MMP)*-2, **(center)** MMP-8, and **(right)** MMP-9 proenzyme levels in untreated abdominal aortic aneurysm controls and doxycycline-treated patients as measured in the protease activity assays. Maximum level of suppression was already found in the low-dose doxycycline group. Results for the higher doses are more variable. *$P < .015$ for MMP-2, $P < .013$ for MMP-8, and $P < .004$ for MMP-9 by Wilcoxon-Mann-Whitney *U* test. Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range *(bars)*.

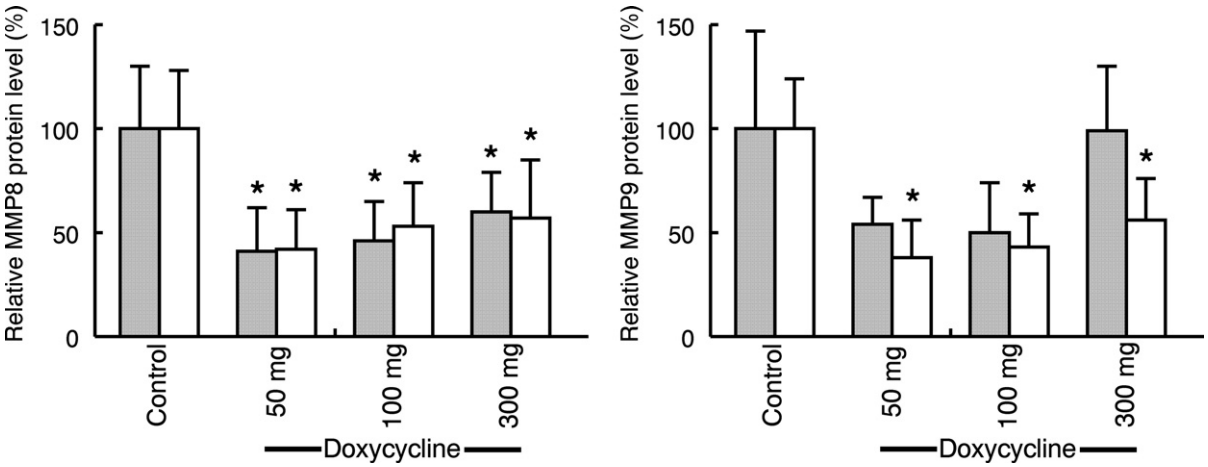

Fig 3. The effect of doxycycline on pro *(gray bars)* and activated *(white bars)* **(left)** matrix metalloproteinase *(MMP)*-8 and **(right)** MMP-9 levels (relative protein levels, mean control, 100%) in untreated abdominal aortic aneurysm controls (n = 5) and doxycycline-treated patients (n = 4 per dose) measured by Western blot analysis. Results are presented with the standard error *(range bars)*. *$P < .020$ by Wilcoxon-Mann-Whitney *U* test.

Doxycycline treatment: protease inhibitor expression. With the exception of a moderate reduction of TIMP2 mRNA expression ($P < .004$), none of the doxycycline doses influenced expression of the protease inhibitors (Supplementary Table II, online only). Yet, as shown in Fig 4, doxycycline therapy increased cystatin C and TIMP1 protein levels (TIMP1 normal-dose and high-dose doxycycline only, $P < .029$).

Doxycycline treatment reduces neutrophil influx in the aneurysmal wall. The rather selective reduction of MMP-8 and MMP-9 (neutrophil collagenase and gelatinase, respectively) protein levels—but not of their mRNA expression—along with reduced mRNA expression of MT6-MMP, the neutrophil-specific MMP-25,[24] suggested that the observed effects of preoperative doxycycline treatment on MMP expression are indirect and may relate to a reduced aortic wall neutrophil content. We quantified the aortic wall neutrophil content by immunohistochemistry (myeloperoxidase staining, Fig 5) that indeed showed that 2 weeks of preoperative doxycycline treatment resulted in a profound reduction of the neutrophil content (Fig 5, $P < .001$). Specificity of myeloperoxidase (Fig 5, *B* and *C*) staining for neutrophils was validated by morphometric analysis of myeloperoxidase-positive cells and absence of an effect of doxycycline on myeloperoxidase mRNA expression and on aortic wall macrophage content (Supplementary Fig, online only). The findings for myeloperoxidase staining were validated by MMP-8 staining, which showed similar results as the myeloperoxidase staining (data not shown).

DISCUSSION

Doxycycline has been shown convincingly to prevent AAA formation in a variety of animal models,[10-13] and the results from two small clinical studies suggest that doxycycline may also reduce AAA expansion in people.[14,15] Remarkably, although the rationale behind doxycycline therapy is based on its putative effects on MMP-9 expression

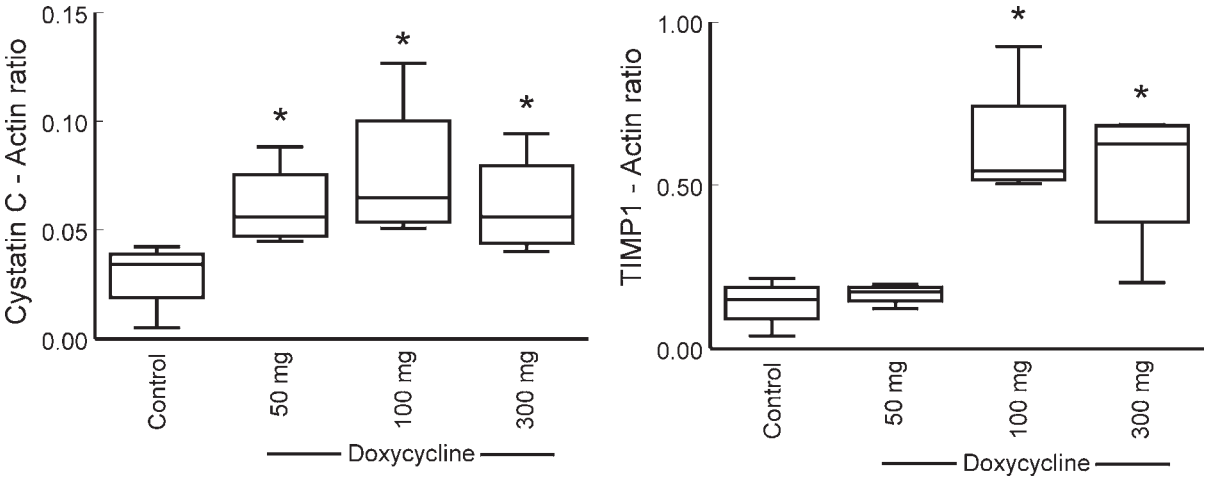

Fig 4. Doxycycline treatment increases protein levels of **(left)** cystatin C, the principle extracellular inhibitor of cysteine proteases, as well as **(right)** tissue inhibitor of metalloproteinase 1 *(TIMP1)* protein levels (100 and 300 mg only). Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range *(bars)*. *$P < .029$ by Wilcoxon-Mann-Whitney *U* test.

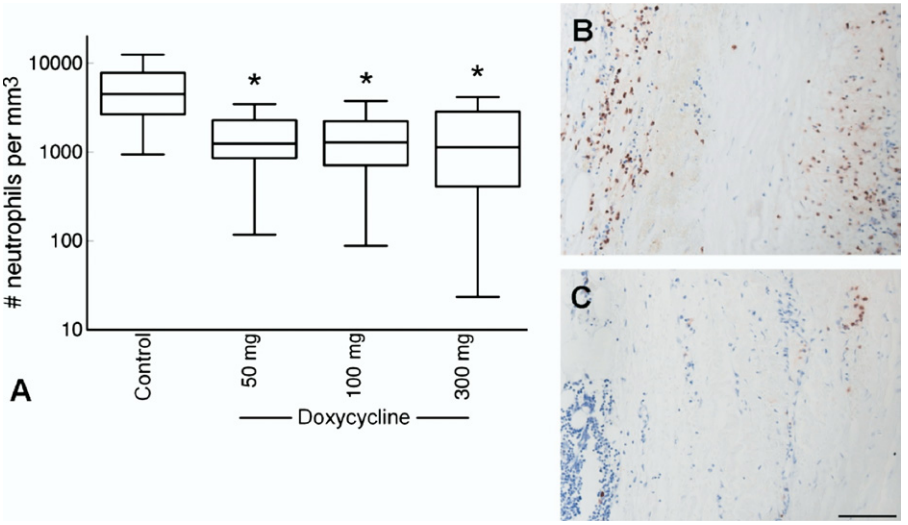

Fig 5. A, Doxycycline reduces neutrophil content in the aneurysmal wall. Results are presented in box plots depicting median value *(horizontal line)*, interquartile range *(box borders)*, and range *(bars)*. *$P < .001$ by Wilcoxon-Mann-Whitney *U* test. **B,** Aneurysm wall neutrophil infiltration (myeloperoxidase staining) in the medial-adventitial border zone in untreated and **(C)** doxycycline-treated individuals. *Bar* represents 100 μm.

and activity,[9] the effects of doxycycline on MMPs in the *human* aneurysm are unclear, with published studies[17,19] suggesting that doxycycline acts through a different mechanism.[16]

In this prospective clinical trial, we examined the effect of three pharmacologically relevant doses of doxycycline—low (50 mg/d), regular (100 mg/d) or high (300 mg/d)—on inflammatory processes in the aneurysmal wall of patients scheduled for elective, open AAA repair in an integrative approach. We first established mRNA expression profiles of the MMP and cysteine collagenases by quantitative real-time PCR. Because this approach does not provide information on the post-transcriptional regulation of protease activity, we applied specific protease activity assays and Western blot analyses to address the post-translational regulation of protease activity, and quantified tissue expression of specific inhibitors of proteases.

Preoperative doxycycline treatment resulted in a selective reduction of MMP-8 and MMP-9 protein levels, as well as suppression of MMP-25 mRNA expression. The effect on MMP-8 and MMP-9 protein levels, but not on

mRNA expression, along with the rather selective effect on MMP-25 mRNA expression,[24] suggests that the effect of doxycycline on protease expression was indirect and may relate to suppression of neutrophil influx. Neutrophil MMP-8 and MMP-9 are both stored (secondary granule) proteins that are temporarily expressed during the late myeloid maturation pathway of neutrophils and are minimally expressed in mature neutrophils.[25,26] This could well account for the apparent discrepancy between the effects of doxycycline therapy on MMP-8 and MMP-9 protein and mRNA expression.

We evaluated a possible effect on neutrophil influx in the aneurysm wall by immunohistochemical staining for myeloperoxidase, the preferred neutrophil marker (Prof D. Roos, Sanquin Research, Amsterdam, The Netherlands, personal, communication) and indeed found that doxycycline treatment profoundly reduced the number of myeloperoxidase-positive cells. The specificity of the myeloperoxidase staining for neutrophils was confirmed by morphometric analysis that showed that all myeloperoxidase-positive cells exhibit a lobed nucleus, typical for neutrophils, and by MMP-8 staining that showed analogous results to the myeloperoxidase staining.

Our immunohistochemical analysis did not indicate myeloperoxidase expression in monocytes and or macrophages.[27] To exclude possible interference by an effect of doxycycline on monocyte/macrophage content or their myeloperoxidase expression, we quantified the number of CD68-positive cells and assessed myeloperoxidase mRNA expression, both of which remained unaffected by doxycycline therapy (Supplementary data, online only).

Our observations are remarkable and suggest that doxycycline may act through an effect on neutrophil infiltration. Neutrophil abundance (presumably resulting from interleukin-8 hyperexpression) is a striking characteristic of AAA disease,[28] and neutrophils may be critically involved in aneurysm formation and growth. Eliason et al[29] and Pagano et al[30] recently showed that abrogated neutrophil influx forestalls aneurysm formation in the elastase model of aneurysm formation, indicating that neutrophil influx is critically involved in aneurysm formation in this established model of aneurysm formation. The pathophysiologic role of the neutrophils in AAA formation and aneurysmal growth is still unclear. Eliason et al[29] showed that the effect occurred independently from MMP-8. Our findings of increased TIMP1 and cystatin C protein levels upon doxycycline therapy provide an alternative explanation: we previously showed that cystatin C deficiency in AAA is secondary and relates to proteolytic degradation by neutrophil-derived proteases.[18] An analogous mechanism has been described for TIMP1,[31] suggesting that neutrophils may unfavorably influence the proteolytic balance through an effect on the inhibitors of protease activity.

Doxycycline has been shown to inhibit neutrophil migration in in vitro studies and studies in healthy volunteers[32]; but to our knowledge, this is the first clinical study showing that doxycycline treatment results in a significantly reduced neutrophil influx in a chronic inflammatory condition such as AAA. The mechanism underlying the reduced neutrophil influx is yet unclear. Tetracyclines, including doxycycline, are known for their pleiotropic immunomodulatory activities that include suppression of inducible nitric oxide synthase and cyclooxygenase-2 expression.[33] Both pathways are implicated in the perpetuation of the inflammatory processes of AAA,[34,35] suggesting that doxycycline treatment may act by attenuation of vascular inflammation. Sugita et al,[36] on the other hand, showed that doxycycline treatment by chelation of intracellular Ca^{++}-ions resulted in a dose-dependent inhibition of neutrophil chemotaxis. Reductions in aortic wall neutrophil content in patients in this study occurred independently of the dose and were already maximal at the sub-antimicrobial dose of doxycycline, suggesting that the effect relates to an anti-inflammatory mechanism.

Independent of its effects on neutrophil influx, doxycycline may further act through direct inhibition of MMP activity (zinc scavenging).[37] Presumably, this effect is dose-dependent and thus more prominent at higher doses. Unfortunately, such binding is lost during the washing steps required in MMP activity assays and not recognizable in Western blot analysis, and we are therefore unable to address the relevance of such an effect.

CONCLUSIONS

Preoperative doxycycline therapy for 14 days resulted in reduced aortic wall neutrophil content that is accompanied by a selective suppression of neutrophil-derived proteases and increased protein levels of the protease inhibitors TIMP1 and cystatin C. Results from this study provide a rationale for the evaluation of doxycycline as a therapeutic means of inhibition of aneurysmal growth. Moreover, our observations argue for a broader application for doxycycline therapy in other pathologies involving neutrophil infiltration and aneurysm formation, such as Kawasaki disease and Behçet disease,[38,39] as well as nonvascular conditions with prominent neutrophil involvement such as Sweet syndrome[40] and chronic obstructive pulmonary disease.[41]

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AUTHOR CONTRIBUTIONS

Conception and design: JHNL, RH, JV, JHvB
Analysis and interpretation: HA, JHNL, RH, JV
Data collection: HA, RG, JHNL
Writing the article: JHNL, HA, RH, JV
Critical revision of the article: JHvB, JHNL
Final approval of the article: HA, JHvB, RH, JV, RG, JHNL
Statistical analysis: JHNL
Obtained funding: JHNL, JHvB, RH
Overall responsibility: JHNL

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